

# Kinase activity in the olfactory bulb is required for odor memory consolidation

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Long-term fear memory formation in the hippocampus and neocortex depends upon brain-derived neurotrophic factor (BDNF) signaling after acquisition. Incremental, appetitive odor discrimination learning is thought to depend substantially on the differentiation of adult-born neurons within the olfactory bulb (OB)—a process that is closely associated with BDNF signaling. We sought to elucidate the role of neurotrophin signaling within the OB on odor memory consolidation. Male mice were trained on odor–reward associative discriminations after bilateral infusion of the kinase inhibitor K252a, or vehicle control, into the OB. K252a is a partially selective inhibitor of tyrosine kinase (Trk) receptors, including the TrkB receptor for BDNF, though it also inhibits other plasticity-related kinases such as PKC and CaMKII/IV. K252a infusion into the OB did not impair odor acquisition or short-term (2 h) memory for the learned discriminations, but significantly impaired long-term (48 h) odor memory (LTM). This LTM deficit also was associated with reduced selectivity for the conditioned odorant in a reward-seeking digging task. Infusions of K252a immediately prior to testing did not impair LTM recall. These results indicate that kinase activation in the OB is required for the consolidation of odor memory of incrementally acquired information.

Memory consolidation requires a complex mobilization of transcriptional activation and regulatory networks culminating in protein synthesis (for review, see Alberini and Kandel 2015). Whereas short-term memory (STM; a few hours duration) can be acquired and behaviorally expressed even when protein synthesis is inhibited (Izquierdo et al. 1999), the stabilization of new memories into forms that can persist for days or years (long-term memory; LTM) depends on these consolidation processes. The relative persistence of these stabilized memories differs widely, and it now is clear that rich networks of molecular interactions, operating at multiple timescales, integrate external experiences with intrinsic mechanisms to govern the persistence of consolidated memories (see Discussion).

Much of what is known regarding the mechanisms of memory formation and consolidation comes from studies based on one-trial fear conditioning, in which performance may depend on hippocampal or amygdalar circuitry. However, most memory-dependent tasks are richer than this paradigm reveals. The routine memories that govern our interactions and define our lives are conditional, context-specific, and statistical in nature, and their utilities vary substantially with both context and timescale. Moreover, the “content” of such a memory, as well as its persistence, must be gradually constructed from statistical estimates of reliability over time and across contexts, typically based on repeated, related experiences. To understand neurophysiological memory mechanisms at this level of complexity will require the development of model systems in which sophisticated behavioral conditioning can be paired with neurophysiological tools capable of measuring and defining the neural representations of these constructed memories (Bieszczad et al. 2013; Weinberger 2015). An important hypothesis is that these memory systems will rely on many of the same underlying molecular mechanisms as those elu-

cidated for one-trial conditioning, although the activation of these signals may be realized on different timescales (Mizuno et al. 2000, 2002).

The olfactory bulb (OB), together with its most closely related structures, is a strong candidate system for the study of iteratively constructed representational memory. Activity patterns in the OB represent the properties of the external chemical environment, including but not limited to the qualities of any odor stimuli of particular interest. Learning shapes the statistical representations defining which patterns of olfactory qualities reliably cue reward (Cleland et al. 2009, 2011), thereby constructing and defining the representations of meaningful “odors” (Cleland 2014). The development, modulation, and persistence of these odor representations depends on OB circuit plasticity (Mandairon et al. 2006, 2008; Guérin et al. 2008; Moreno et al. 2012; Vinera et al. 2015; see Discussion). To more directly assess whether OB-dependent odor learning depends on molecular mechanisms similar to those elucidated in hippocampal fear conditioning, we here demonstrate that the consolidation of incremental, appetitive odor learning depends upon kinase activation within the olfactory bulb at the time of encoding.

## Results

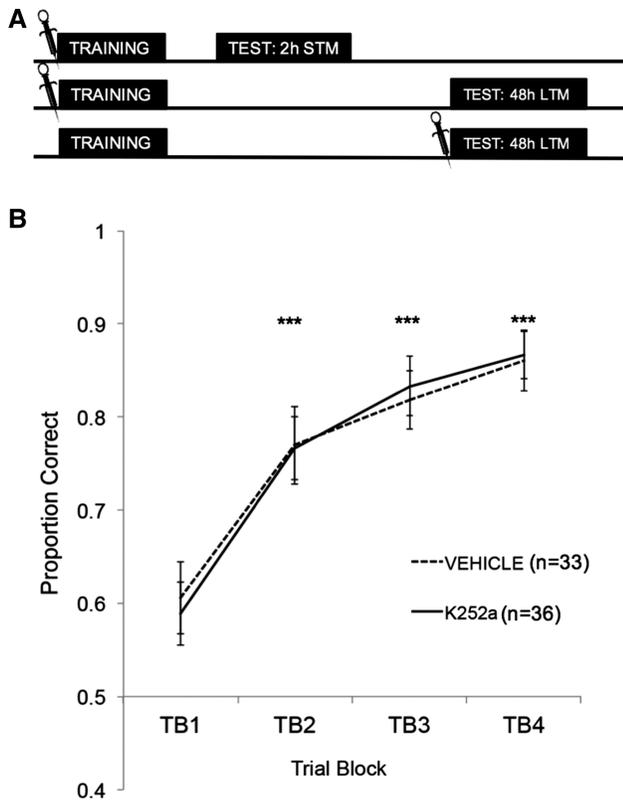
### Vehicle and K252a-infused mice show no differences in rate of acquisition

First, we analyzed learning curves during the training phase (Fig. 1) to identify any effects of K252a infusion on the rate of acquisition. We ran a linear mixed model with two fixed effects, “infusion” and

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**Figure 1.** (A) Timelines for training, testing, and infusions. The *top* two lines show timelines for the main experiment (Figs. 2, 3). The *bottom* line shows the timeline for the retrieval control (Fig. 4). (B) Learning curves during the training phase. Odor training comprised 20 consecutive trials, divided into four consecutive trial blocks (TB) of five trials each for analysis. Each trial was scored as correct (mouse dug in the rewarded odor first) or incorrect (mouse dug in the unrewarded odor first); the five scores in each trial block were averaged to generate a proportion-correct metric. Mice with OB infusions of K252a (solid line) or vehicle (dotted line) did not differ in their rates of acquisition of the odor-reward association. Asterisks indicate significant increases in proportion correct compared to TB1 for both vehicle and K252a cohorts ( $P \leq 0.001$  for all comparisons).

“trial block” (TB); “mouse” and “odor set nested within mouse” were random effects. We observed a significant main effect of “trial block” ( $F_{(3,183.692)} = 43.735$ ,  $P < 0.001$ ), but no effect of “infusion” ( $F_{(1,85.685)} = 0.132$ ,  $P = 0.717$ ) and no significant interaction of infusion and trial block ( $F_{(3,183.692)} = 0.111$ ,  $P = 0.954$ ). Post hoc tests, using the Šidák adjustment for multiple comparisons, confirmed that the drug and vehicle groups did not differ on any of the trial blocks ( $P > 0.05$  for all comparisons). In addition, TB2, TB3, and TB4 were significantly higher than TB1 for all comparisons ( $P \leq 0.001$  in all cases), indicating that both groups successfully learned the association. These results show that K252a infusion did not affect the rate of acquisition of the odor-reward association.

### K252a infusion into OB disrupts long-term, but not short-term, odor memory

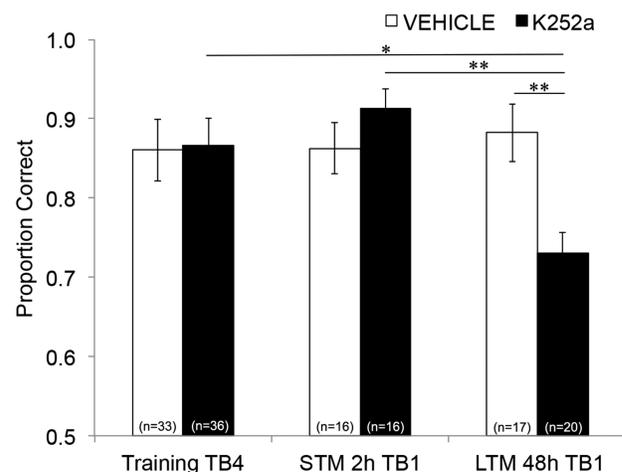
We compared odor memory performance between groups to assess whether STM and/or LTM depended on intact Trk receptor activity within the olfactory bulb. Specifically, we compared the proportion correct during the last trial block of the training phase (Fig. 2; TB4) to those during the first trial blocks (TB1) of short-term (2 h latency) and long-term (48 h latency) odor memory testing.

We ran a linear mixed model with two fixed effects: infusion and trial block (TB4<sub>tr</sub>, TB1<sub>2h</sub>, and TB1<sub>48h</sub>). Mouse and odor set nested within mouse were random effects. Our analysis showed a significant interaction between infusion and trial block ( $F_{(2,77.558)} = 4.043$ ,  $P = 0.021$ ), with no significant main effects of infusion ( $F_{(1,55.629)} = 1.438$ ,  $P = 0.236$ ) or trial block ( $F_{(2,69.979)} = 1.360$ ,  $P = 0.263$ ).

Post hoc pairwise comparisons with Šidák correction further showed that vehicle-infused mice retained a stable odor memory at both 2 h (STM) and 48 h (LTM) post-training ( $P > 0.05$  for all comparisons with training performance). In contrast, while K252a-infused mice exhibited no memory deficit after 2 h compared to the end of training ( $P > 0.05$ ), their task performance was significantly reduced after 48 h ( $P = 0.018$ ). Moreover, performance at 48 h post-training also was significantly reduced compared to performance at 2 h ( $P = 0.009$ ), and to the performance of vehicle-infused control animals at 48 h post-training ( $P = 0.006$ ). There was no difference in short-term (2 h) memory performance between vehicle- and K252a-infused mice ( $P = 0.356$ ). This pattern demonstrates that K252a inhibition of kinase receptors within olfactory bulb selectively disrupted long-term, but not short-term, odor memory.

### K252a infusion disrupts long-term memory for odor selectivity

Whereas the “proportion correct” measure quantifies the accuracy of decision making based on odor memory, the “selectivity index” (SI; see Materials and Methods), which is based on digging time measurements, enables assessment of the relative certainty with which those digging decisions were made (based on the premise that greater certainty underlies greater perseverance; Cleland et al. 2009). We computed this SI for five “probe trials” (trials 1, 5, 10, 15, and 20) in which earned rewards and removal from the



**Figure 2.** Impairment of long-term memory, but not short-term memory, in K252a-infused mice. The graph depicts the mean proportion correct of the last trial block (TB4) of the training phase and the first trial blocks (TB1) of both the STM and LTM testing phases. Trial blocks each comprise five sequential trials as described in Figure 1. All mice were given intrabulbar infusions of K252a (black bars) or vehicle (white bars) prior to training. Groups were then tested either 2 h (STM) or 48 h (LTM) after the end of training. Post hoc comparisons showed that mice infused with K252a showed significantly impaired LTM (comparison with training TB4;  $P = 0.018$ ) but not STM ( $P > 0.05$ ). LTM performance of K252a-infused mice also was significantly lower than the STM performance of K252a-infused mice ( $P = 0.009$ ), and lower than the LTM performance of vehicle-infused mice ( $P = 0.006$ ).

test chamber were delayed for 1 min (see Materials and Methods). Probe trials were administered during both the training and testing phases.

Linear mixed model analysis was performed on SI data with four fixed effects: “training/testing,” “STM/LTM,” infusion (K252a/vehicle), and probe trial (T1, T5, T10, T15, T20). Mouse and odor set nested within mouse were included as random effects. The analysis yielded highly significant main effects of training/testing ( $F_{(1,711)} = 66.433$ ,  $P < 0.001$ ) and probe trial ( $F_{(4,711)} = 51.046$ ,  $P < 0.001$ ), respectively, indicating that the mean SI differed between the training and testing phases (Fig. 3, cf. A and B) and that the SI across all groups differed over the five probe trials T1–T20. There also was a highly significant two-way interaction between training/testing and probe trial ( $F_{(4,711)} = 11.147$ ,  $P < 0.001$ ), indicating that the SI trajectory across the five probe trials differed significantly between the training and testing phases.

Pairwise testing (using false discovery rate correction for 780 comparisons) demonstrated that, in the training phase, the SI at T20 was significantly greater than that at T1 for all four training groups (STM/K252a, STM/vehicle, LTM/K252a, LTM/vehicle;  $P < 0.001$  in all cases; Fig. 3A; note that during the training phase there was not yet any experimental distinction between the STM and LTM cohorts). During the test phase, in contrast, the SI at T20 was significantly greater than that at T1 only for the LTM/K252a group ( $P < 0.001$ ), but not for any other cohort ( $P > 0.05$  in all cases; Fig. 3B). As illustrated in Figure 3B, this result indicates that the latter groups retained near-asymptotic SI performance after training (2 or 48 h). In contrast, the LTM cohort infused with K252a had lost the benefit of training (T1), although it was able to swiftly regain performance levels comparable to the other groups after a few additional trials (T5+). Specifically, the LTM/K252a cohort was significantly less selective during test trial T1 than was the LTM/vehicle cohort ( $P < 0.001$ ), indicating that K252a infusion was directly responsible for the long-term memory deficit. The LTM/K252a cohort also was significantly less selective than the STM/K252a cohort ( $P = 0.016$ ), indicating that the amnesic effects of K252a infusion into OB were specific to long-term memory—

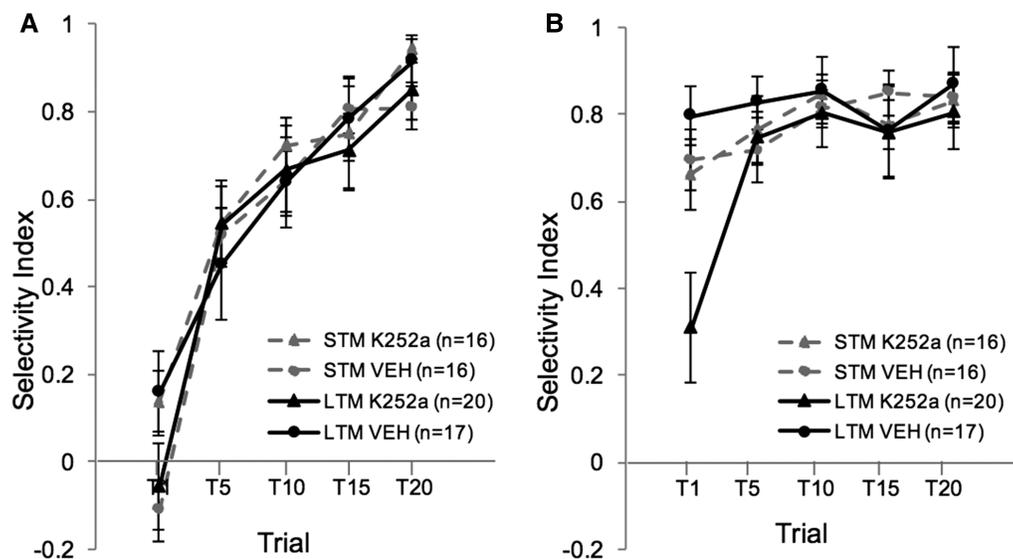
that is, to memory consolidation—corroborating the results of Figure 2.

### K252a infusion does not affect memory retrieval

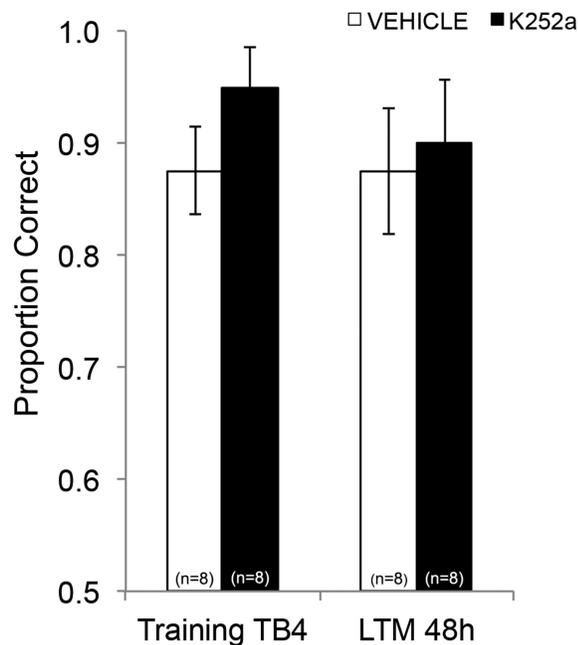
To ensure that the observed LTM deficits were not due to delayed effects on memory retrieval, we ran a separate control experiment in which mice received infusions of K252a or vehicle immediately prior to LTM testing (Fig. 4). No infusions were given prior to training. To compare performance during the last block of training trials to that during the first block of test trials (after 48 h), we ran a linear mixed model with training/testing and infusion as fixed effects; mouse and odor set nested within mouse were random effects. We found no significant main effects of training/testing ( $F_{(1,14)} = 0.055$ ,  $P = 0.817$ ) or infusion ( $F_{(1,14)} = 1.361$ ,  $P = 0.263$ ), and no significant interaction ( $F_{(1,14)} = 0.592$ ,  $P = 0.454$ ), indicating that the observed LTM deficits did not result from disruptions to memory retrieval. This finding suggests that the deficits in long-term odor memory observed after pretraining infusions of K252a into the olfactory bulbs resulted specifically in disruption of memory consolidation mechanisms.

### Discussion

We here demonstrate that K252a infusion in the olfactory bulb during encoding blocks the consolidation of incremental, appetitive odor memory. K252a is a kinase inhibitor that inhibits the Trk family of neurotrophin receptors (Tapley et al. 1992), among other plasticity-related kinases including protein kinase C and calcium/calmodulin-dependent protein kinases II and IV (Kase et al. 1986; Gschwendt et al. 1996; Yoshida et al. 2000). While the broad effects of this inhibitor do not enable us to specify which of these pathways underlie the observed memory deficit, we suggest that the effects of K252a in this study are mediated primarily by TrkB receptor inhibition. Anatomically, TrkB immunoreactivity and mRNA hybridization are observed in all layers of the OB (Deckner et al. 1993; Bergami et al. 2013). Functionally, TrkB has



**Figure 3.** K252a infusion specifically impairs long-term memory for odor selectivity. (A) Selectivity indices (SI) for all groups during the five probe trials of the training phase (trials 1, 5, 10, 15, and 20; see Materials and Methods); note that there is not yet any experimental distinction between STM and LTM groups. No differences in SI among groups were observed during training. (B) Selectivity indices for all groups during the five probe trials of the testing phase. All groups retained full memory for the selectivity learned during training except for the K252a-infused group at a 48 h delay (LTM). The LTM/K252a group was significantly impaired in its memory-dependent performance during T1 ( $P < 0.001$  compared to LTM/vehicle), but regained an odor selectivity comparable to that of other groups by T5.



**Figure 4.** K252a infusion has no effect on memory retrieval. A separate cohort of mice was trained normally and then tested 48 h later (LTM). All animals were given infusions of K252a or vehicle into the olfactory bulb immediately prior to the testing phase. No differences were observed in the proportion correct between K252a-infused and vehicle-infused animals, indicating that blockade of Trk receptors in the OB did not affect retrieval ( $P > 0.05$  for all main effects and interactions). Timeline for training, testing, and infusions shown as bottom line of Figure 1A.

been implicated in the dendritic branching of mitral and tufted cells (Imamura and Greer 2009). BDNF activation of TrkB receptors also has been shown to regulate the dendritic development and spine proliferation of adult-born interneurons in the OB and their synaptic integration into OB circuits with mitral/tufted cells (Gascon et al. 2005; Bergami et al. 2013; McDole et al. 2015). Notably, experience-dependent plasticity within the OB and OB-dependent odor memory both depend on the integration of these new neurons (Alonso et al. 2012; Arruda-Carvalho et al. 2014; Huang et al. 2016). Conversely, odor learning increases the survival of adult-born neurons in the OB (Rochefort et al. 2002; Kermen et al. 2010). In contrast, TrkA expression has not been observed in the OB (Roskams et al. 1996). TrkC is expressed in the OB, but to date has been associated primarily with the expression of its ligand NT-3 within primary OSNs and the anterograde transport of NT-3 into OB glomeruli (Liu et al. 2013). Moreover, inhibition of NT-3/TrkC signaling has not been elsewhere associated with the disruption of consolidation; for example, cortical infusion of NT-3 (and NGF) antisense oligonucleotides had no effect on long-term memory in chicks, whereas BDNF antisense disrupted its formation (Johnston and Rose 2001). Consequently, while a contributing effect of TrkC inhibition cannot be ruled out, concerted expression and functional studies favor TrkB receptor inhibition as the primary mediator of the olfactory memory consolidation impairment observed here.

The observed dependence of odor memory consolidation on kinase receptor activation within OB, here attributed to TrkB receptor activation by BDNF, is wholly consistent with the profile of BDNF expression and dependence exhibited by consolidation in other memory systems, based on both one-trial and incremental learning paradigms. For example, BDNF is expressed in the hippocampus immediately following one-trial inhibitory avoidance

learning (IA, a form of fear conditioning), and initiates a positive feedback cascade resulting, among other effects, in substantial cAMP response element binding protein (CREB) activation (specifically, increases in pCREB levels) as well as fear memory consolidation (Bambah-Mukku et al. 2014). Blocking the effects of this early BDNF expression disrupts LTM, but not STM, for the fear event, and infusion of human recombinant BDNF (hrBDNF) into the hippocampus rescues the LTM amnesia that can be caused by glucocorticoid receptor inhibition (Chen et al. 2012). Later, after initial consolidation, hippocampal BDNF levels depend on the intensity of the original unconditioned stimulus (US; footshock), and in turn determine the persistence of the fear memory (Bekinschtein et al. 2008); elevated hippocampal BDNF levels in this “delayed stabilization” phase (at 12 h, but not 9 or 24 h, post-conditioning) are both necessary and sufficient for the extended maintenance of the fear memory. During this phase, blockades of protein synthesis (by hippocampal infusion of anisomycin) or BDNF activity (by hippocampal infusion of anti-BDNF antibodies or antisense oligonucleotides) induced amnesia at 7 d, but not 2 d, post-training (Bekinschtein et al. 2007, 2008), whereas infusion of human recombinant BDNF (hrBDNF) into the hippocampus 15 min after this anisomycin infusion reversed the impairment and restored 7-d fear memory (Bekinschtein et al. 2008). Moreover, these 12-h BDNF manipulations interacted with the effects of unconditioned stimulus intensity. Low-amplitude footshocks normally induced fear memories for 2 d that could be extended to 7 d by hrBDNF infusion, whereas high-amplitude footshocks normally induced 7-day fear memories that were limited to 2 d by hippocampal BDNF blockade (Bekinschtein et al. 2008). That is, well beyond the timescale of the first few hours post-event during which consolidation mechanisms construct long-term memories, additional molecular processes operate over a timescale of many hours to (at least) days, governing the persistence of these long-term memories. Indeed, the active processes of memory stabilization and maintenance may continue indefinitely (Miyashita et al. 2008).

One-trial fear conditioning is a powerful tool with which to study the molecular dynamics of memory, but of course most natural learning arises from milder US consequences that are less clearly predictable by salient conditioned stimuli (CSs) or configurations thereof. Accordingly, most learning is incremental, and ultimately statistical in nature; that is, the predictive CS configurations must be elucidated and learned along with the associated US consequences. It has long been known that the consolidation of incremental forms of learning also is susceptible to protein synthesis inhibition, and that the extent and forms of this susceptibility vary over the course of learning (Flexner et al. 1962, 1963; Hernandez and Abel 2008; Peng and Li 2009). Interestingly, in contrast to one-trial IA tasks, in which pCREB levels in the hippocampus rose immediately after conditioning (Bernabeu et al. 1997; Taubenfeld et al. 1999), hippocampal pCREB levels increased only after multiple successive days of training on an appetitive, hippocampus-dependent radial maze task (Mizuno et al. 2000, 2002). Moreover, on this task, reference memory and working memory performance remained dependent on BDNF even after four weeks of training. On day 28, memory performance was asymptotic, yet after four subsequent days of continuous hippocampal infusion of antisense BDNF oligonucleotides, the rats exhibited significantly reduced radial maze performance (Mizuno et al. 2000). That is, the overall sequence of events and vulnerabilities underlying the consolidation and maintenance of radial maze performance memory resembles that governing fear memory consolidation and maintenance, except for substantial differences in timescale. We here show that appetitive odor learning based on olfactory bulb plasticity also appears to depend on similar processes. How these molecular mechanisms regulate the integration of

multiple corroborating experiences into a common, statistically predictive memory remains to be investigated.

Natural memories have many properties aside from persistence, of course. In studies of perceptual memory, generalization gradients can be used to assess the breadth, or specificity, of a CS—that is, how much variance in the quality of the CS will still be associated with the consequential US (Shepard and Chang 1963; Shepard 1987). The repeated pairing of an odor CS with reward progressively sharpens the olfactory generalization gradients around that CS (Cleland et al. 2009), iteratively updating the internal representation (from a relatively broad prior) so as to better estimate the actual quality variance of the reward-predicting odor (Cleland et al. 2011). This learned specificity can fade with time; in one olfactory generalization study, the sharp gradients obtained by massed odor–reward pairings progressively flattened over a timescale of hours (Tong et al. 2014), though we here show that task parameters can substantially extend the persistence of conditioned odor memories. Importantly, these learned perceptual gradients depend on olfactory bulb circuitry, as pharmacological manipulations of the olfactory bulb systematically affect the selectivity of rats' responses to learned odors (Mandaïron et al. 2006, 2008) as well as the properties of short-term and long-term odor memory (Guérin et al. 2008; McNamara et al. 2008; Kermen et al. 2010; Moreno et al. 2012; Vinera et al. 2015). Olfactory bulb circuitry is highly plastic in response to odor learning (Huang et al. 2016); in particular, the formation and maintenance of long-term memory in the olfactory bulb appear to depend upon adult neurogenesis—the ongoing migration and recruitment of new interneurons into bulbar circuitry (Moreno et al. 2009, 2012; Kermen et al. 2010; Sultan et al. 2010; Mandaïron et al. 2011; Lepousez et al. 2013, 2014; Arruda-Carvalho et al. 2014).

Accordingly, the olfactory system, and the olfactory bulb in particular, comprises a powerful system in which to study the properties and dependencies of naturalistic, representational, statistical learning at a neural circuit level. Like the hippocampus, the olfactory bulb exhibits adult neurogenesis, rich structural plasticity (Matsutani and Yamamoto 2004; Berghuis et al. 2006; Imamura and Greer 2009; McDole et al. 2015), task-dependent synaptic plasticity (Huang et al. 2016), and long-term synaptic potentiation (Gao and Stowbridge 2009). Its particular additional strength, however, is that measurable odor representations are systematically transformed by learning in both persistence and form (e.g., generalization gradients), enabling study of how statistical learning affects the *content* of memories, as assessed both behaviorally and neurophysiologically. To develop this approach requires investigation of whether learning in the olfactory bulb depends on molecular regulatory systems similar to those studied in hippocampus, amygdala (Rattiner et al. 2004, 2005), and other plastic circuits. We here show that the consolidation of reward-conditioned odor memories depends on kinase activity in olfactory bulb, consistent with a role for BDNF signaling similar to that which it performs in hippocampal memory consolidation.

## Materials and Methods

### Animals

A total of 27 adult male CD-1 mice (Charles River), 8 wk old at the time of cannulation, were used in this study. All procedures were performed under the auspices of a protocol approved by the Cornell University Institutional Animal Care and Use Committee (IACUC). Cornell University is accredited by The Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International).

The mice were kept on a 12:12 h reverse light/dark cycle and allowed free access to water at all times. For the duration of the behavioral experiments, they were kept on a food-restriction

schedule designed to maintain them at ~90% of their free feeding weight. This food restriction schedule began 3 d prior to the beginning of behavioral tasks.

### Olfactory bulb cannulation

Mice were anesthetized with gaseous 4% isoflurane (Henry Schein) in pure oxygen and secured into a stereotaxic apparatus (Kopf Instruments). For the duration of the surgery, mice were maintained under 1.5%–2% isoflurane anesthesia through a nose cone. Guide cannulae (26-gauge; PlasticsOne) were inserted into both olfactory bulbs using the following coordinates with respect to bregma: AP +5.0 mm, ML  $\pm$ 0.75 mm, and DV 1.0 mm. Two screws also were drilled into the skull over the cerebellar formation to provide an anchor for the dental cement cap. Dental cement was used to secure the guide cannulae to these screws and to cover the incision area. Dummy plugs were placed into the guide cannulae to prevent blockage. For 2 d after the surgeries, mice were fed softened food and given injections of ketoprofen (0.2 mg/kg) and saline. Animals recovered for at least 7 d before beginning behavioral training.

### Associative discrimination task

#### Apparatus

Animals were tested in a clear Plexiglas cage (28 cm long  $\times$  17 cm wide  $\times$  12 cm high) with a removable opaque black center divider separating a “test chamber” from a “resting chamber.” A mouse was placed into the resting chamber at the beginning of each session. Each trial was initiated by removing the divider to enable the mouse to enter the test chamber; trials ended by returning the mouse to the resting chamber.

#### Infusions

Animals received OB-specific infusions of the tyrosine kinase receptor inhibitor K252a (50  $\mu$ M; Sigma-Aldrich K2015; 5% DMSO in saline) or vehicle (5% DMSO in saline). K252a is a partially selective antagonist for the Trk family of neurotrophin receptors (Tapley et al. 1992), including the TrkB receptor that is preferentially activated by brain-derived neurotrophic factor (BDNF; for review, see Andero et al. 2014; see Discussion). Mice were awake and free to move (not anesthetized) during the infusions. Infusions were delivered bilaterally into the OBs (2.0  $\mu$ L final volume per bulb; 0.2  $\mu$ L/min infusion rate; 10 min total infusion time). Injectors were left inside cannulae for an additional 5 min after infusion to prevent backflow and facilitate intrabulbar diffusion. Mice received infusions immediately before training, except for one control experiment in which mice were given infusions only prior to testing. Figure 1A shows the timelines of training, testing, and drug infusions for the main experiment of Figures 2–3 (top two lines) and the retrieval control experiment of Figure 4 (bottom line).

#### Odor sets

For shaping prior to the experiment, we used  $\pm$ -limonene (Sigma-Aldrich) as the rewarded odor and plain mineral oil as the unrewarded odor. All odorants were diluted in mineral oil so as to emit a theoretical steady-state vapor phase partial pressure of 1.0 Pa (Table 1; Cleland et al. 2002) and mixed into play sand at a ratio of 400  $\mu$ L diluted odorant per 100 g sand. For the training and testing phases, five separate odor pairs were used: butanoic acid/pentanoic acid; hexanal/heptanal, propyl acetate/butyl acetate; 2-octanone/2-heptanone; and pentanol/hexanol. Each odor pair consisted of two odorants with the same functional groups that differed from each other by one carbon in the aliphatic chain; accordingly, the odorants in each pair are moderately perceptually similar (Cleland et al. 2002, 2009). For training and testing, one odor of each pair was chosen as the rewarded odor and the other was unrewarded; this assignment was counterbalanced to mitigate any innate odor preference biases (Devore et al. 2013).

**Table 1.** Odor pairs used in behavioral tasks

Odor set	Odor 1	Odor 2
1	Pentanoic acid 225.1	Butanoic acid 63.6
2	Hexanal 11.1	Heptanal 35.3
3	Propyl acetate 3.1	Butyl acetate 10.9
4	2-Octanone 87.4	2-Heptanone 28.7
5	Pentanol 37.2	Hexanol 127.3

Numbers indicate the corresponding volumes ( $\mu\text{L}$ ) of each odorant diluted into 50 mL mineral oil to obtain theoretical vapor-phase partial pressures of 1.0 Pa (Cleland et al. 2002).

All mice were trained and tested on multiple odor pairs in order to increase statistical power while reducing the use of animals and ensure that results were not dependent on the use of particular odorants. Twenty-three animals were used for the main study (Figs. 1–3); each was trained and tested repeatedly using different odor pairs from Table 1. To improve counterbalancing, individual animals were rotated into different experimental groups (infusion, test latency) when tested with different odor pairs. Consequently, the  $n$  values reported in the figures reflect animal/odor set combinations and do not report the number of animals *per se* that contributed to each training/testing group. Accordingly, all statistical analyses included “individual mouse” and “odor set nested within mouse” as random effects.

In the retrieval control experiment (Fig. 4), four additional mice were trained and tested with four odor sets from Table 1 (pentanoic acid/butanoic acid; hexanal/heptanal, propyl acetate/butyl acetate; and pentanol/hexanol). The reported results are the average of training and testing data for four different odor sets with the same group of mice. The infusion received by a given mouse alternated between sessions to control for repeated drug exposure. Statistical analyses again included individual mouse and odor set nested within mouse as random effects.

### Shaping

The mice underwent a 10-d behavioral shaping period prior to the start of the experiment. Animals were brought into the procedure room and handled for 10 min per day for the first 2 d following recovery from surgery (Days 1 and 2). On Day 3, a petri dish (Pyrex, 60 mm diameter, 15 mm height) filled with play sand (Quikrete) scented with  $\pm$ limonene was placed into the home cages of the animals. These dishes were filled with 10–15 5-mg sucrose reward pellets (PJ Noyes Precision Pellets; TestDiet). The sand and pellets were replenished on Day 4. On Days 5–6, the mice were acclimated to the behavioral apparatus. Two dishes of scented sand (one with limonene and one with mineral oil), were placed into the Plexiglas test chamber without the center divider; ten reward pellets were mixed into the limonene-scented dish. Each mouse was placed into the test chamber for 10 min, and was allowed to explore freely and to consume the reward pellets.

On Day 7, the mice were introduced to a shortened version of the final testing procedure. Again, two dishes were placed into the behavioral apparatus, including the center divider. A single reward pellet was placed on top of the limonene-scented sand, and the mouse was placed into the resting chamber. The center divider was lifted and the mouse was allowed to enter the test chamber and retrieve the reward pellet. Animals were returned to the resting chamber either after they retrieved the reward pellet or after 5 min elapsed. This was repeated for 10 trials. On any given trial, the rewarded dish was randomly placed on the left or right according to a random number generator. This procedure was repeated on Day 8, except that on Day 8 the reward pellet was buried progressively more deeply with each trial. All mice were digging for an unseen reward pellet by the tenth trial on Day 8.

On Day 9, animals were presented with the full 20-trial version of the task. As on Day 8, reward pellets were fully buried under the sand in the dish; however, each trial lasted only 1 min. On this day, animals were allowed to dig freely in both dishes for the re-

ward. On Day 10, the animals underwent the same 20 trials, but they were not permitted to self-correct; if a mouse dug in the unrewarded dish (scented with mineral oil) first, they were returned to the resting chamber and the next trial began. Mice that dug first in the limonene-scented sand were allowed to retrieve the reward pellet before being returned to the resting chamber.

### Training and testing

Once the mice would reliably dig for an unseen, odor-cued reward pellet, we began the experiment. The training phase began 2 d after shaping was completed. To test the main hypothesis, intrabulbar drug/vehicle infusions were delivered immediately prior to training. (For the memory retrieval control experiment, infusions were delivered immediately prior to the testing phase instead). Training began immediately following infusions. Each mouse was placed into the resting chamber. Two dishes of sand scented with a novel odor pair (Table 1) were placed in the test chamber; one of the dishes also contained a buried reward pellet. During each trial, the barrier was lifted and the mouse entered the test chamber. Training comprised 20 trials in total; in 15 of the 20 trials (see below), if the mouse dug in the unrewarded odor dish first, it was immediately returned to the resting chamber; if it dug in the rewarded odor first, it was allowed to retrieve the sugar reward and then returned to the resting chamber. The next trial then began immediately.

Testing was then performed either 2 h (STM) or 48 h (LTM) following training. The procedures, the odor pair, and the reward-associated odor were the same as those used for training in each mouse. The reward-associated odor within each pair was counterbalanced among mice. Figure 1A shows the timelines of training, testing, and drug infusions for the main experiment of Figures 2–3 (top two lines) and the retrieval control experiment of Figure 4 (bottom line).

For both the training and testing phases, the reward pellet was omitted from the dishes on Trials 1, 5, 10, 15, and 20 to enable measurement of the mouse’s persistence in reward seeking. On these trials, animals were allowed to dig freely in either dish, including self-correction; digging times in each of the two dishes were recorded over the course of 1 min (the maximum trial duration). At the end of the trial, if the animal had dug in the rewarded odor first, a reward pellet was surreptitiously dropped into that dish. If the animal dug in the unrewarded odor first, it was returned to the resting chamber without reward.

### Statistical analysis

The specific statistical design for each analysis can be found in the Results. In general, analyses were performed on two dependent measures: the *proportion correct* among initial choices and a *selectivity index* (SI) based on digging persistence. First, on a given training or test trial, a “1” was assigned to trials in which the mouse dug in the rewarded odor first, and a “0” if the mouse dug first in the unrewarded odor. We excluded data from mice that did not dig at all on 10 consecutive trials during a session with a given odor set (either training or testing). Four mice failed to meet this performance criterion during one of their sessions; the resulting data were excluded from the final analysis. Blocks of five trials were averaged together to create a “proportion correct” measure across four “trial blocks” (i.e., trial block 1 was the average of trials 1–5, trial block 2 comprised trials 6–10, etc.). Second, the SI was computed by dividing the difference between digging times in the rewarded and unrewarded odors (“rewarded–unrewarded”) by the sum of those times. Hence, SI values close to 0 indicated that a mouse dug non-selectively, whereas SI values close to +1 indicated a strong preference for digging in the rewarded odor. SIs were computed only for Trials 1, 5, 10, 15, and 20 within both the training and testing phases.

We performed linear mixed effects analyses on these transformed measures using IBM SPSS 22.0 and R 3.4.1. Because the dependent measures were not continuous, unbound variables, and thereby violate two assumptions for linear models, we performed a logit transformation prior to statistical analysis. Specifically, we

first linearly rescaled the SI values  $[-1,1]$  to  $[0,1]$ , then replaced all data values ( $X$ ) of 0 with 0.01 and values of 1 with 0.99, and finally transformed them using the formula  $\ln(X/(1-X))$ . The fixed effects differed for each analysis, and are described in the Results. To compensate for intrinsic performance differences and any potential effects of using multiple odor sets, all analyses also included random effects for individual mouse and odor set nested within mouse. For analysis of proportion post hoc tests on significant interactions identified by the full model; Šidák correction was used to compensate for multiple pairwise comparisons in post hoc testing. For selectivity index data, post hoc tests were corrected for multiple comparisons using the false discovery rate.

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